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DYSENTERY VACCINE STIMULATING AN IMMUNE RESPONSE AGAINST SHIGATOXIN, PLASMIDS AND HOST STRAINS FOR IT

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Dysentery vaccine stimulating an immune response against Shiga toxin, plasmids and host strains for it

Current strategies to develop effective vaccine candidates against shigellosis caused by *Shigella dysenteriae* 1 strains aim to induce protective immune responses against surface antigens, in particular the lipopolysaccharide somatic (O-) antigen, of the bacterium, and involve either the construction of hybrid attenuated *Salmonella* strains expressing the O-antigen of *Shigella dysenteriae* 1 (Mills et al. 1988; Mills and Timmis 1988) or the construction of attenuated strains of *Shigella dysenteriae* 1 (Fontaine et al. 1990). Oral administration of such vaccines should stimulate a localized mucosal immune response which should prevent infection of intestinal mucosa by immune exclusion of the pathogen. *Shigella dysenteriae* 1 strains produce a potent toxin, Shiga toxin (for reviews see, Tesh and O'Brien 1991; Jackson 1990; Sekizaki and Timmis 1988; O'Brian and Holmes 1987; Cantey 1985) a bipartite molecule consisting of two distinct types of subunit: the A-subunit (Mr = 32,000) and the B-subunit (Mr = 7,700) which associate noncovalently with an apparent subunit stoichiometry of one A-chain and five B-chains (Olsnes et al. 1981; Donohue-Rolfe et al. 1984). The B-subunit binds to its cell surface receptor which is a glycolipid, globotriosyl ceramide (Gb3) carrying a terminal Gal $\alpha(1-4)$Gal moiety (Jacewicz et al. 1986; Lindberg et al. 1987). The A-subunit inhibits eucaryotic protein synthesis by acting as an N-glycosidase that cleaves an adenine residue at nucleotide position 4324 of the 28S rRNA of the 60S ribosomal subunit (Endo et al. 1988; Saxena et al. 1989). Chromosomal *stx*A and *stx*B genes which encode the Shiga toxin A- and B-subunits, respectively, have been mapped, cloned (Sekizaki et al. 1987; Strockbine et al. 1988) and sequenced (Strockbine et al. 1988). The role of Shiga toxin in pathogenesis has recently been elucidated by Sansonetti and colleagues (Fontaine et al. 1988) who demonstrated that it influences the severity of bacillary dysentery by inducing colonic ischemia and inflation of the polymorphonuclear intestinal compartment during the infectious process. The toxin can also damage the vascular endothelium of kidneys and it has been suggested that it may be the cause of the severe complications such as haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (Richardson et al. 1988; Karmali 1989). It would therefore seem desirable that vaccine candidates against *S. dysenteriae* 1 infections should also stimulate an immune response against the Shiga toxin.

Therefore, it was the problem of the invention to provide a Dysentery vaccine which stimulates an immune response against Shiga toxin.

This problem has been solved by providing a vaccine comprising microorganisms expressing the Shiga toxin B subunit or fragments or derivatives of the said B subunit. Especially, the construction of plasmids that would stably encode for Lam B hybrid proteins carrying various regions of the Shiga toxin B subunit and their expression in E. coli K-12 and *aroA*-derivatives and in attenuated *Salmonella typhimurium aroA*-antigen carrier vaccine strains SL 3235 and SL 3261 (Hoseith and Stocker 1981) is preferred.

LamB is an *E. coli* outer membrane protein involved in the entry of maltose and maltodextrins into the cell (Szmelcman and Hofnung 1975) and also serves as a surface receptor for several bacteriophages, including bacteriophage lambda (Charbit and Hofnung 1985). Extensive genetic and protein structure characterization of LamB, has led to the identification of a unique site between amino acids 153 and 154 (a region in the cell surface exposed loop of the protein) where insertion of foreign epitopes can lead to the exposure of the inserted polypeptide on the bacterial cell surface (Boulain et al. 1986; Charbit et al. 1986; Charbit et al. 1988). As a result, the LamB protein has been proposed as a carrier protein for the delivery of heterologous epitopes by whole bacteria to the immune system. With a view to producing *S. typhimurium aroA*-strains expressing LamB::B-subunit hybrid proteins, which when used as live immunogens would

elicit specific B-subunit immune responses and antibodies directed against the epithelial cell receptor binding site of the B-subunit and thereby neutralize the endocytosis and hence the biological activity of the Shiga toxin.

MATERIALS AND METHODS

Bacterial strains, plasmids and media.

The bacterial strains and plasmids used in this study were as follows: *E. coli* strain pop6510 (*thr. leu. ton*B, *thi. lac*Y1, *rec*A, *dex*5, *met*A, *sup*E) and plasmid pAJC264, which carries *lam*B gene under the control of the *tac* promoter and is inducible with isopropyl-ß-D-thiogalactopyranoside (IPTG) (Boulain et al. 1986), were a kind gift from M. Hofnung (Inst. Pasteur, Paris). *Salmonella typhimurium aro*A- mutants SL3235 (*aro*A, *crr*-401), SL3261 (Hoseith and Stocker 1981) and the restriction negative *S. typhimurium* strain SL5283 were kindly provided by B.A.D. Stocker (Stanford Univ. Sch. of Medicine, USA). Plasmid pDB74 carries a mutated Shiga toxin locus with a transposon Tn-mini-Kan insertion in the *stx*A gene (Brazil et al. 1988). *E. coli* strain JM83 (*ara*, (*lac-pro*AB), *rps*L, π80, *lac*Z M15) (Yanisch-Perron et al. 1985), phagemid pGC1 (Milton et al. 1986), plasmid pcon1 (Lorenzo et al. 1987) which carries the *lac*Z gene under the control of the aerobactin promoter and *E. coli* DH5 strain (*end*A1, *rec*A1, *hsd*R17, *sup*E44, *thi*-1, , *gyr*A96, *rel*A1, F') were kindly provided by Victor de Lorenzo. Plasmid pJLA503 carrying thermoinducible c1857 gene (Schauder et al. 1987) was a gift of JEG McCarthy and *E. coli aro*A- strain AB2829 (O'Gaora et al. 1989) was a kind gift of G. Dougan. Purified LamB protein and polyclonal anti-LamB antiserum were kind gifts from M. Hofnung.

Luria broth and Luria agar (Miller 1972) were used as complete media for the routine growth of all strains and X-gal/IPTG (5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside and Isopropyl-ß-D-thiogalactopyranoside respectively) plates were prepared as previously described (Miller 1972). Where appropriate, bacterial growth media were supplemented with ampicillin (100 ug/ml) or kanamycin (50 ug/ml). Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow enzyme) and all other enzymes were purchased either from Boehringer GmbH (Mannheim, Germany) or from New England Biolabs, Inc., Beverly, Mass.; and were used in accordance with the recommendations of the manufacturer. Chemicals and salts were purchased from Sigma

Chemical Co. (St. Louis, Mo.).

DNA manipulations.

Preparation and handling of DNA was according to standard protocols (Maniatis et al. 1982). Bacterial transformation was performed as described by Hanahan (1983) and DNA sequencing was done by the dideoxynucleotide chain-termination method (Sanger et al. 1977). Oligonucleotides were synthesised with an Applied Biosystems model 380B DNA synthesizer and purified on OPC columns (Applied Biosystems Inc.). Oligonucleotide-directed *in vitro* mutagenesis was performed as described previously (Kunkel 1985) using the Bio-Rad Mutagene Phagemid *in vitro* mutagenesis kit. Polymerase Chain Reaction (PCR) was carried out using the method described by Saiki and colleagues (1988).

SDS-polyacrylamide gel electrophoresis.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Schägger and Jagow (1987). SDS-PAGE prestained molecular weight markers were either from Bio-Rad (in kD; 97.4, 66.2, 45.0, 31.0, 21.5, 14.4) or from Sigma (in kD; 84.0, 58.0, 48.5, 36.5, 26.6) as indicated in the figure legends.

Purification of B-subunit protein.

Overnight bacterial cultures grown at 30°C were diluted to OD_{560} = 0.4 and incubated further until the OD_{560} reached 0.8. The culture was heat-induced at 42°C for 4 hours and the cells were harvested by centrifugation (7000 rpm for 15 min.). The pellet was washed twice in 10 mM Tris-HCl (pH 8.0) and resuspended in a solution containing 25% sucrose w/v, 1mM Na_2EDTA, 10 mM Tris-HCl pH 8.0; and gently shaken for 10 min. at 30°C. The cells were harvested and rapidly resuspended in ice cold distilled water (osmotic shock treatment) and shaken gently for 10 min. at 4°C. After centrifugation the supernatant fraction (periplasmic fraction) was collected and the pellet was resuspended in 10 mM Tris-HCl pH 8.0 (cytoplasmic fraction). Cracking buffer was added to both fractions and the samples were boiled for 5 min. prior to SDS-PAGE. Protein concentration was determined by the method of Lowry (Lowry et al. 1951) with BSA as the standard. FPLC purification.....

Western blotting of total cellular extracts.

This was carried out essentially as described by Boulain et al. (1986). Briefly, the bacterial strains were grown at 37°C in Luria broth supplemented with ampicillin. At OD_{600} = 0.7, IPTG was added to a final concentration of 10^{-3} M and the culture was grown further for 45 min. The cells were collected by centrifugation and resuspended at a 20-fold concentration in water. Total cellular extracts (20 ul) from each strain were diluted to a final volume of 40 ul loading buffer (LB; 60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue, 1 ul ß-mercaptoethanol), heat denatured for 5 min. at 100°C and run on SDS-PAGE. The proteins were transferred to nitrocellulose filter using a semi-dry electroblotter, and LamB/B-subunit fusion proteins were identified using either the preabsorbed B-subunit specific polyclonal antiserum or with one of the two B-subunit specific monoclonal antibodies. The antigen-antibody complexes were revealed with iodinated protein-A, followed by autoradiography.

Antibody production.

Monoclonal antibodies.

Four 6-week old female BALB/c mice were immunized at 3 week intervals with the FPLC purified B-subunit (100 - 200 ug protein/injection) using Freund's complete adjuvant for the first injection and incomplete adjuvant for the two subsequent injections. Sera were tested by ELISA and Western blotting. The spleen cells from the mouse giving the strongest reaction were fused with the myeloma line X63Ag8 (Kearney et al. 1979). Colony supernatants were screened by ELISA using 96-well microtiter plates, wells were coated with approximately 0.5 ug of purified B-subunit. Positive supernatants were further tested by Western blotting and cloned twice by limiting dilution.

Polyclonal antibodies.

Two rabbits were immunized with the FPLC-purified recombinant B-subunit (200 - 400 ug protein/injection) using standard immunization procedures. Sera were further purified by repeated absorption on *E. coli* K-12 or anti-B-subunit antibodies were isolated by affinity chromatography on the B-subunit coupled to CNBr-activated sepharose 4B. Specific antibodies were eluted with 0.2 M sodium acetate (pH 2.7).

Immunoelectron Microscopy.

Postembedding labeling

The different strains were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentration) in nutrient broth for 1 h on ice. After three washing steps with PBS (0.1M phosphate buffer, 0.9% NaCl, pH 6.9) containing 10 mM glycine for blocking free aldehyde groups, cells were embedded by the progressive lowering of temperature method (Roth et al. 1981) using Lowicryl K4M resin with slight modifications: a) infiltration step with 1 part ethanol/ 1 part K4M resin was performed overnight, b) infiltration step with 1 part ethanol/ 2 parts K4M resin was done for 12 h, and c) infiltration with pure K4M resin was for 2 days with several changes of the resin mixture. After polymerisation of the samples at -35°C ultrathin sections were incubated with the protein A-purified cross-adsorbed antibody (180 ug IgG protein/ml) for 16 h at room temperature and washed with PBS, followed by incubation of the sections with protein A-gold complexes (PAG, gold-particle size 10 nm, concentration A_{520} nm= 0.02). The PAG complexes were produced following established procedures (Slot and Geuze 1981). Subsequently, the sections were rinsed with PBS containing 0.01% Tween 20 and then with distilled water. After air-drying the sections were poststained with 4% aqueous uranyl acetate, pH 4.5, for 5 min.

Whole cell labeling

Cells were harvested by centrifugation and resuspended in 0.5 ml PBS. They were then adsorbed onto freshly prepared collodium-covered nickel grids, rinsed with distilled water and air-dried. Grids were incubated on drops of the cross-adsorbed and protein A-purified antibody (90 ug IgG protein/ml) for 30 min at room temperature, washed by a mild spray of PBS from a plastic bottle and subsequently incubated protein A-gold complexes (PAG, 10 nm gold-particle size, concentration A_{520} nm= 0.01) for 10 min at room temperature. Grids were subsequently rinsed with PBS containing 0.01% Tween 20, then with distilled water and air-dried.

Electron microscopy

Samples were examined with a Zeiss electron microscope CEM 902 or 10 B at an acceleration voltage of 80 kV and at calibrated magnifications.

Indirect immunofluorescence microscopy.

Over night bacterial cultures were washed twice with PBS and resuspended in PBS to an approximate density of 10^9 cells/ml. The following procedure was performed at room temperature. Glass coverslips (diameter 12 mm) were coated for 5 min. with poly-lysine (MW 80 kD; 1 mg/ml in distilled water) washed twice with PBS and incubated with bacteria (50 ul bacterial suspension/ coverslip) for 15 min. in a wet chamber. After 2 washes with PBS the coverslips were incubated for 1 hr with 10 ul of either the polyclonal B-subunit antiserum (1:1000 diluted in PBS-10% fetal calf serum) or the monoclonal B-subunit antibody (undiluted hybridoma supernatant). Coverslips were washed three times with PBS and incubated for 1 hr with 10 ul of affinity-purified fluorescein-conjugated rabbit anti-mouse or sheep anti-rabbit IgG's (1:50 diluted in PBS-10% Fetal calf serum). After 3 washes with PBS coverslips were imbedded with Moviol and analysed by immunofluorescence microscopy using a Zeiss microscope.

Immunization and collection of sera and intestinal washings.

Oral immunization was carried out essentially as described by Clements and colleagues (1986). Briefly, for primary immunization with *S. typhimurium aro*A- strains expressing various LamB/B-subunit proteins, 8-10 week old female BALB/c mice were immunized (four mice per immunization) orally with two doses containing 10^{10} CFU of each strain on days 0 and 4. Twenty-one days post primary immunization a booster was given and mice were sacrificed a week later. Oral immunization of mice was carried out with the aid of a feeding tube and intestinal fluid and serum were collected from orally immunized mice and the serum collected from i.p. immunized mice.

Inocula for immunization were prepared as follows;

Strains carrying *tac* promoter based plasmids were grown as O/N cultures in Luria broth supplemented with ampicillin. The cells were then grown fresh in the same medium until OD_{600} reached 0.7. IPTG was added (final concentration of 1mM; to induce the *tac* promoter) and the cells were grown further for 45 minutes. The cultures were washed twice in sterile normal saline and resuspended in an appropriate volume of saline to obtain a final concentration of 10^{11} CFU/ml. 0.1 ml cell suspension was used for oral immunization. Cells were further diluted to 10^6 CFU/ml and 0.1 ml cells were used for i.p. immunization.

Strains carrying ß-lactamase promoter based plasmids were grown as above until OD_{600} reached 1.0. The cells were washed, resuspended and used for immunization as mentioned above. Strains carrying aerobactin promoter based plasmids were also grown as above until OD_{600} reached 0.3. 2,2'-bipyridyl was added (final concentration of 100 uM; for induction of aerobactin promoter) and cells were grown further for 3 hr. The cultures were washed, resuspended and used for immunization as stated above.

ELISA.

Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.2). For anti-B-subunit determinations, microtiter plates were precoated with 1 ug of purified B-subunit. Serum anti-B-subunit IgG+IgM was determined using peroxidase conjugated goat anti-mouse IgG+IgM (purchased from Dianova) and mucosal B-subunit IgA was determined using peroxidase conjugated goat anti-mouse IgA (obtained from Southern Biotechnology Inc.). The results were read using the Bio-Rad Microplate Reader (Model 3550).

Specifically, two different N-terminal regions of the Shiga toxin B-subunit along with the complete B-subunit have been inserted in the cell surface exposed loop of the LamB protein and the expression of the hybrid proteins has been analysed using three different promoter systems i.e. (i) *in vitro* inducible *tac* promoter providing high level expression. (ii) *in vivo* inducible aerobactin promoter and (iii) a modified synthetic ß-lactamase promoter providing moderate level constitutive expression. Hybrid protein expression studies have been carried out in *E. coli*, *Salmonella typhimurium* and attenuated antigen carrier strains of *S. typhimurium aro*A- mutants and the hybrid vaccine strains have been used to immunize mice by the oral and intra-peritoneal routes. The results show that there is a membrane export defect in the *S. typhimurium aro*A- mutants which blocks the membrane secretion of LamB and its derivatives. High level expression of hybrid proteins using the *tac* promoter proved deleterious to the vaccine strains and greater stability was achieved using the ß-lactamase and aerobactin promoters. Immunization studies revealed significant B-subunit specific mucosal and serum antibody responses suggesting that this expression system could be incorporated in attenuated vaccine strains designed to protect against infections caused by *Shigella dysenteriae* 1 inorder to stimulate anti-Shiga toxin immune responses thereby reducing the severity of the disease.

Plasmid construction for high level expression and purification of B-subunit.

The 1.2 kb *Ssp*I fragment from plasmid pDB74 (Fig. 1), which carries the complete B-subunit gene (*stx*B) and some flanking DNA, was subcloned into the Klenow filled *Eco*RI site of plasmid pJLA503. The resulting plasmid pSU108 carries the *stx*B gene under the control of the lambda P_L and P_R promoters (in the native *stx* operon the *stx*B region is transcribed from a promoter upstream of *stx*A). Uninduced and induced (with IPTG) whole cell extracts of strain DH5/pSU108 were electrophoresed on SDS-PAGE; two molecular weight forms of the B- subunit were expressed at a high level (Fig. 2.1, Lanes C and D). It was presumed that the higher molecular weight polypeptide was the premature form of the B-subunit while the lower molecular weight form represents the mature form of the protein. After osmotic shock treatment of the cells, a procedure which would be expected to release the periplasmic contents, the pellet and supernatant samples were electrophoresed on SDS-PAGE. As expected, the pellet was enriched for the higher molecular weight protein (Fig. 2.1, lane E), indicating that it was cytoplasmically

localized, while the lower molecular weight polypeptide was found in the supernatant (Fig. 2.1, Lane F). FPLC purification of the supernatant fraction followed by SDS-PAGE analysis (Fig. 2.1, lane G) showed that the purified sample contained the mature B-subunit protein.

B-subunit specific polyclonal and monoclonal antibodies.

The purified B-subunit was used to immunize rabbits and mice to raise B-subunit specific polyclonal and monoclonal antibodies respectively. The polyclonal antiserum was further purified by repeated adsorption with whole cells of *E. coli* K-12 carrying the plasmid vector pJLA503. The purity of the antiserum was assessed by western blotting (Fig. 2.2, Lanes A and B). Two monoclonal antibodies StxBMb1 and StxBMb2 were identified, purified and were shown by western blot analysis to react specifically with the B-subunit (Fig. 2.3, lanes A and B; data shown only for StxBMb1).

Construction of LamB::B-subunit gene fusions.

Three different regions of the mature B-subunit sequence were selected to generate LamB fusions, namely (i) the complete B-subunit (69 amino acids; studies to assess size limitations of foreign polypeptides that can be stably inserted into LamB revealed that 70 amino acid inserts formed the upper limit; larger insertions were found to be unstable and toxic to the host cells; Charbit et al. 1988). (ii) the N-terminal 27 amino acids; Harari and colleagues (1988) have previously shown that antibodies directed against synthetic peptides of the N-terminal 26 amino acid region of the Shiga toxin B-subunit neutralized the cytotoxicity, enterotoxicity and neurotoxicity of Shiga toxin to varying degrees. It has also been shown that synthetic peptides representing the C-terminal region also elicit protective responses in mice (Harari and Arnon 1990), although this region was not analysed in our study. (iii) a 17 amino acid region (positions 11 to 27 of the mature protein); a Hopp and Woods (1981, 1983) hydrophobicity/hydrophilicity plot of the B-subunit sequence showed that the first 10 amino acids constitute a hydrophobic region and it has been suggested that such polypeptides inserted into LamB often lead to toxicity since they may act as "stop transfer" sequences and lead to a block in the export process (Charbit et al. 1988).

As an intermediate step prior to the generation of *lam*B::*stx*B gene fusions it was necessary to create *Bam*HI and *Bgl*II sites at appropriate positions in *stx*B to permit excision of precise fragments of *stx*B and their insertion into the *Bam*HI site of *lam*B to generate in-frame fusions. The Muta-gene phagemid *in vitro* mutagenesis kit from Bio-Rad was used to create these sites. The *Bam*HI/*Sal*I fragment carrying the entire *stx*B gene from plasmid pSU108 (Fig. 1) was subcloned between the *Bam*HI/*Sal*I sites of phagemid pGC1 (Fig. 1). The resulting phagemid, pSU109, was then used for site-directed mutagenesis. Synthetic oligonucleotide StxB-N (Table 1) was made which carries a *Bam*HI site just before the first codon (Thr) of the structural part of the *stx*B gene. A second primer, StxB-C (Table 1) carries a *Bgl*II site downstream of the penultimate codon (codon 68 = Phe) of *stx*B. The generation of this site eliminates the *stx*B stop codon (TGA) and also replaces the last codon of *stx*B (+69 = Arg) by a serine residue. Phagemid pSU109 was mutagenized using primers StxB-N and StxB-C to give phagemid pSU110 (Fig. 1) which carries the newly created *Bam*HI and *Bgl*II sites flanking the *stx*B structural gene. A third oligonucleotide StxB-3 (Table 1) was synthesised which carries a *Bgl*II site downstream of the 27th amino acid codon of *stx*B and replaces the 28th amino acid (Glu) by Asp. This primer was used in conjunction with primer StxB-N to mutagenize phagemid pSU109 and the resulting phagemid pSU111 (Fig. 1) carried the *Bam*HI site as described for phagemid pSU110 and a *Bgl*II site downstream of the 27th amino acid codon of *stx*B. All the sites generated in phagemids pSU110 and pSU111 were confirmed by DNA sequencing. Phagemids pSU110 and pSU111 were cleaved with *Bam*HI/*Bgl*II and the B-subunit inserts were subcloned into the *Bam*HI site of plasmid pAJC264. Subclones were screened by restriction digests to identify those in the correct orientation. The resulting plasmids pSU112 and pSU113 (Fig. 1) were found to carry the complete B-subunit (designated *lam*B-*stx*B) and the 27 amino acid region (designated *lam*B-*stx*27B) respectively. Nucleotide sequencing of the fusion junctions revealed that the sequences were as expected. The plasmid pSU113 was digested with *Bam*HI and *Acc*I, blunt-ended using DNA polymerase (Klenow enzyme) and religated. This resulted in plasmid pSU114 (Fig. 1) which carries the 17 amino acid region fused to LamB (designated *lam*B-*stx*17B). Each of these plasmids were transformed into the *E. coli* host pop6510 and the SL3235 *aro*A- strain of *Salmonella typhimurium*. Prior to transformation of SL3235, the plasmids were first passaged through the restriction negative *S. typhimurium* strain SL5283.

Western blotting analyses of the expression of the LamB/B-subunit hybrid proteins in E. coli K-12 and in Salmonella typhimurium aroA- strain SL3235.

Whole cell extracts were prepared from both *E. coli* pop6510 and *S. typhimurium* SL3235 strains (before and after IPTG induction) carrying plasmids pSU112 (*lam*B-*stx*B), pSU113 (*lam*B-*stx*27B) and pSU114 (*lam*B-*stx*17B) along with control strains carrying plasmid pAJC264 (*lam*B) and these extracts were electrophoresed on SDS-PAGE.

Western blotting analysis of the gels using the preabsorbed B-subunit polyclonal antiserum showed (Fig. 3) that in uninduced cells the LamB/StxB protein could not be detected (Figs. 3A and B, lanes 3) but the LamB/Stx27B and LamB/Stx17B proteins were observed (Figs. 3A and B, lanes 5 and 7 respectively). After induction, all 3 hybrid proteins were detected in large amounts (Figs. 3A and B, lanes 4, 6 and 8 respectively) and some degradation of proteins was also found since the background in induced cells was higher than in uninduced or control cells. The results were similar in *E. coli* and *Salmonella typhimurium* hosts.

Immunoelectron microscopy analysis of the expression of LamB::B-subunit hybrid proteins in various E. coli and Salmonella typhimurium strains.

In order to examine whether or not the B-subunit regions were stably expressed as LamB hybrids with the B-subunit regions exposed on the bacterial cell surface, immunoelectron microscopy studies of whole cells and of thin bacterial sections were carried out (Fig. 4). The B-subunit regions were revealed using pre-absorbed polyclonal B-subunit specific antiserum.

Thin section studies showed that in *E. coli* K-12 strains expressing LamB/Stx17B and LamB/Stx27B, the hybrid protein was localized in the cytoplasmic compartment and in the bacterial membrane (Fig. 4.1 g and h). The LamB/StxB protein however could only be seen in the cytoplasmic compartment as aggregated molecules suggesting instability of expression (Fig. 4.1 f). In *Salmonella typhimurium aro*A- (SL3235) no cytoplasmic aggregates of the LamB/StxB protein were found and all the fusion proteins were only cytoplasmically localized (Fig. 4.2 f, g and h).

Whole cell analysis of the same strains showed that in *E. coli* K-12, all fusion proteins except for LamB/StxB, exposed the respective B-subunit regions on the bacterial cell surface (Fig. 4.1 b, c and d). In *Salmonella typhimurium aro*A- (SL3235) the B-subunit regions of none of the hybrid

proteins were exposed on the bacterial cell surface (Fig. 4.2 b, c and d).

These results raised the question of whether the *aro*A- mutation or whether the *Salmonella typhimurium* host were responsible for the observed membrane export defect in SL3235. In order to resolve these questions the recombinant and control plasmids were transformed into *S. typhimurium* SL5283 (with no defect in *aro* genes), SL3261 (another *S. typhimurium aro*A- mutant) and AB2829 (*E. coli* K-12 *aro*A- strain). The hybrid strains were analysed by immunoelectron microscopy as described above. The results with SL5283 and AB2829 hosts were identical to those in *E. coli* K-12 (Figs. 4.3 and 4.4) while SL3261 host gave the same results as those observed for SL3235 (data not shown).

The strains mentioned above were also simultaneously analysed using anti-LamB serum and the results (summarized in Table 2) showed that in control and hybrid strains of *E. coli* K-12, *E. coli aro*A- and *S. typhimurium* SL5283 the LamB protein could be detected on the bacterial cell surface. However, neither of the *S. typhimurium aro*A- mutants had LamB exported to the outer membrane.

These results suggested that LamB and LamB hybrids could be expressed in *S. typhimurium* in a way similar to that found in *E. coli* K-12 (compare Figs. 4.3 and 4.1 respectively) and also that the *aro*A- defect was not responsible for the membrane export defect since *E. coli aro*A- strain could also export the LamB hybrids to the outer membrane (compare Figs. 4.4 and 4.1). It is possible that the *S. typhimurium aro*A- strains SL3235 and SL3261 have accumulated secondary mutations which lead to the membrane export defect.

Indirect Immunofluorescence analysis of expression of LamB::B-subunit hybrid proteins in E. coli K-12 and in S. typhimurium aroA- strain.

In order to analyse whether or not the B-subunit regions in LamB hybrids could be detected on the surface of whole cells without the harsh treatment employed in electron microscopy studies, the *E. coli* K-12 and *S. typhimurium aro*A- strains harbouring the hybrid LamB::B-subunit plasmids were analysed by indirect immunofluorescence using the pre-absorbed B-subunit specific polyclonal antiserum (Fig. 4.5; data shown only for plasmids expressed in *E. coli* K-12). In both phase-dark and phase-light *microscopy*, *E coli* K-12, *E. coli aro*A- and *S. typhimurium* (SL5283) cells expressing the LamB/Stx17B and LamB/Stx27B proteins, the B-subunit regions

could be detected on the bacterial cell surface. However only the phase-light cells of the strains harbouring the LamB/StxB protein could be detected by immunofluorescence. This suggested that the LamB/StxB protein was localized in the cytoplasmic compartment and could be detected only when the cells had lysed. In the case of *S. typhimurium aro*A- SL3235 and Sl3261 strains none showed any fluorescence except for phase-light cells. These results were in complete agreement with those obtained using immuno-electron microscopy.

Synthesis of a modified ß-lactamase promoter and test for function.

The *tac* promoter in plasmid pAJC264 gave rise to two major difficulties: (i) upon induction with IPTG the insert DNA is expressed at very high levels and the resulting protein product(s) eventually prove toxic to the cells. This made it very difficult to grow bacteria harbouring the hybrid plasmids to 10^8 live cells/ml of culture. For oral immunization of BALB/c mice at least 10^8 live *S. typhimurium aro*A- are required (J. Clements; personal communication). (ii) The *tac* promoter is inducible with IPTG which makes it difficult to obtain expression *in vivo*.

We therefore chose to replace the *tac* promoter with (i) a modified synthetic ß-lactamase promoter which provides moderate level, constitutive expression and (ii) an *in vivo* inducible promoter, the aerobactin promoter, which is induced under iron limiting conditions as found in intestinal tissues.

Fig. 5A shows the DNA sequences of the ß-lactamase promoter and a modified, synthetic version of it (Fig. 5B) which includes (i) an *Eco*RI site upstream of the -35 region and a *Bam*HI site following the ATG translation start codon, (ii) *Xho*I and *Nde*I sites flanking the ribosome binding site which permits replacement of the translation initiation region depending on the required promoter strength. (iii) removal of 10 bp from the translational initiation region primarily inorder to reduce the size of the oligonucleotides to be synthesised.

The *Eco*RI/*Bam*HI fragment in plasmid pcon1 (Fig. 6) was replaced with the *Eco*RI/*Bam*HI fragment carrying the ß-lactamase promoter to give plasmid pSU208. This plasmid was transformed into strain JM83 and the colonies were plated on X-gal/IPTG plates. All colonies obtained were blue suggesting that the synthetic ß-lactamase promoter was functional and expressed ß-galactosidase.

Replacement of tac promoter in LamB and LamB=B-subunit plasmids with ß-lactamase and aerobactin promoters.

A problem encountered in an attempt to replace the *tac* promoter was that in plasmid pAJC264 no convenient restriction enzyme sites were found between the *tac* promoter and the ATG translation start codon of *lam*B. A fragment encoding part of the signal sequence of *lam*B was synthesised as a *Bgl*II/*Cla*I PCR fragment (Table 1, oligonucleotides LamB-8 and LamB-9 used for PCR) and was used to replace the *Bam*HI/*Cla*I fragment of plasmid pSU208 (Fig. 6). The resulting plasmid pSU115, was cleaved with *Eco*RI/*Cla*I and the ß-lactamase promoter (including the PCR fragment) was used to replace the respective *Eco*RI/*Cla*I fragments in plasmids pAJC264 (*lam*B), pSU112 (*lam*B/*stx*B), pSU113 (*lam*B/*stx*27B) and pSU114 (*lam*B/*stx*17B). The resulting plasmids pSU116, pSU117, pSU118 and pSU119 respectively (construction of pSU116 is shown in Fig. 6).

A similar approach was used to replace the *tac* promoter with the aerobactin promoter. The *Bam*HI/*Bgl*II *stx*B fragment from plasmid pSU110 (Fig. 6) was inserted into the *Bam*HI site of plasmid pcon1 to give plasmid pSU207. The *Bgl*II/*Cla*I PCR fragment was subcloned into the *Bam*HI/*Cla*I sites of plasmid pSU207 to give plasmid pSU120. The *Eco*RI/*Cla*I fragment from plasmid pSU120 was used to replace the respective fragments from plasmids pAJC264, pSU112, pSU113 and pSU114 to give plasmids pSU121, pSU122, pSU123 and pSU124 respectively (construction of pSU121 is shown in Fig. 6).

Western blotting analyses of expression of LamB/B-subunit hybrids under the control of either the modified ß-lactamase or aerobactin promoters.

Plasmid pSU207 (Fig. 6) harboured in *E. coli* K-12 and *Salmonella typhimurium aro*A- strains was analysed for expression of B-subunit=ß-galactosidase fusion protein to determine if the aerobactin promoter was functional. Fig. 7 shows the data for the expression of B-subunit=ß-galactosidase fusion protein in both *Salmonella typhimurium aro*A- strains (SL3235 and SL3261; data for *E. coli* K-12 not shown). In all strains, the fusion protein was expressed after induction with dipyridyl (Fig. 7, lanes 4 and 6) suggesting that the aerobactin promoter was functional. Some residual activity was also found in uninduced cultures (Fig. 7, lane 5).

The expression of LamB and LamB::B-subunit hybrids in plasmids pSU116, pSU117, pSU118 and pSU119 (Fig. 6) was analysed by western blotting of whole cell bacterial extracts using either the LamB or the B-subunit polyclonal antisera. Fig. 8A shows that LamB and LamB::B-subunit fusion proteins could be detected in plasmids pSU116, pSU118 and pSU119 (Fig. 8A, lanes 2, 3 and 4 respectively) but not in pSU117 (Fig. 8A, lane 5). However in the latter strain a background of smaller polypeptides could be observed suggesting that the LamB/StxB protein was probably expressed but is degraded due to its toxic nature as earlier observed for plasmid pSU112. Similar results were obtained using the B-subunit polyclonal antiserum (Fig. 8B).

Fig. 9 shows the results obtained in similar western blotting analysis for plasmids pSU121, pSU122, pSU123 and pSU124 where the expression of proteins under the control of aerobactin promoter was analysed. All plasmids except for pSU122 were found to express LamB and LamB/B-subunit fusion proteins after induction with dipyridyl.

B-subunit specific immune responses in mice after oral and intra-peritoneal immunization with hybrid Salmonella typhimurium aroA- strains expressing LamB::B-subunit fusions.

Salmonella typhimurium aroA- strain SL3261 carrying plasmids expressing LamB::B-subunit fusion proteins under the control of either the *tac*, aerobactin or the modified synthetic ß-lactamase promoters were used to immunize BALB/c mice by the oral or intra-peritoneal (i.p.) routes. The intestinal fluid and serum of mice immunized orally and the serum of mice immunized intra-peritoneally was analysed for B-subunit specific antibody responses.

The results showed (Figs 10.1, 10.2 and 10.3) that in all cases, significant B-subunit specific antibody responses were detected. The immunization experiments were repeated and it was consistantly observed that the *tac* promoter based plasmids were difficult to control and although 10^5 live bacterial cells/ml of innoculum were used for i.p. immunization a significant proportion of dead and lysed cells had accumulated in the innoculum. Immunization with these bacteria often killed the mice within 24 hours presumably due to endotoxin shock caused by LPS released from lysed cells. The aerobactin and ß-lactamase promoter based constructs were significantly better although in some cases the high level expression of hybrid protein still lead to the accumulation of lysed cells in the innoculum. This lead to death of mice within 24 hours of primary immunization.

The results also showed that the antibody responses in general increased with increasing size of the B-subunit region expressed in the LamB constructs i.e. antibody responses increased in the order of LamB/Stx17B, LamB/Stx27B and LamB/StxB.

DISCUSSION.

Several different approaches are currently being adopted to induce humoral and mucosal antibody responses to protein antigens from bacterial and viral pathogens. They involve either the use of peptides conjugated to carrier molecules for immunization (Harari et al. 1988) or the expression of hybrid proteins where the polypeptide of interest is fused to carrier molecules such as flagellin (Newton et al. 1989, 1990; Stocker 1990), ß-galactosidase (Brown et al. 1987; Jacob et al. 1985), LamB (references listed in O'Callaghan et al. 1990), cholera toxin B-subunit (Sanchez et al. 1990), heat-labile toxin B-subunit (Schödel and Will 1989; Clements and Cárdenas 1990), *E. coli* alkaline phosphatase (Freimuth and Steinman 1990), TraT lipoprotein (Harrison et al. 1990) and *E. coli* fimbrae (Klemm and Hedegaard 1990).

In this study, three different regions of the Shiga toxin B-subunit have been fused in the bacterial cell surface exposed loop of the LamB protein. Expression studies showed that the LamB/Stx17B and LamB/Stx27B but not LamB/StxB hybrid proteins express the B-subunit regions on the bacterial cell surface of *E. coli* K-12, *S. typhimurium*, and *E. coli aro*A-strains. However, in none of the cases was the B-subunit region expressed on the cell surface of *S. typhimurium aro*A-strains SL3235 and SL3261. *S. typhimurium* also has its own LamB protein (Palva and Westermann 1979; Schülein and Benz 1990) and it has been shown that monoclonal antibodies raised against LamB of *E. coli*, are immunologically cross-reactive with LamB of *S. typhimurium* (Bloch and Desaymard 1985). However, in the electron microscopy and indirect immunofluorescence studies using LamB antiserum only the *E. coli* K-12, *E. coli aro*A- and *S. typhimurium* SL5283 hosts exhibited LamB the bacterial cell surface (Table 2).

The nature of the defect in *S. typhimurium aro*A- strains SL3235 and SL3261 which leads to a block in the export of LamB and the hybrid proteins to the bacterial outer membrane is not clear. The possibility that the *aro*A mutation may be the cause was ruled out by the observation that the export of the hybrid proteins to the outer membrane occurred in an *E. coli aro*A- strain. It is possible that the *S. typhimurium aro*A- strains have accumulated secondary mutations which

may lead to the defect in the export process. These strains are however not defective in the transport of the outer membrane proteins normally found in the wild-type *S. typhimurium* and hence it seems unlikely that any of the "chaperone" proteins of the Sec-mediated protein translocation pathway (for reviews on Sec pathway see; Swidersky et al. 1990; Bassford et al. 1991) are mutated since these mutations are usually lethal to the cell. Recent studies aimed at the dissection of the process of LamB export into membrane targeting and transmembrane translocation steps showed (Swidersky et al. 1990) that apart from the proteins involved in the Sec pathway of protein export, other unidentified factor(s) are also involved such as a protease sensitive membrane receptor. It is possible that one or more of such factors may have undergone mutations in these strains.

Although it has been shown that foreign epitopes need not be expressed on the bacterial cell surface (Leclerc et al. 1990) in order to stimulate an immune response to the inserted epitope(s), in the case of LamB hybrids, expression of the foreign polypeptide at the bacterial cell surface is one of the indices to suggest that the hybrid protein is stably expressed with respect to its proper folding, transport and assembly in the outer membrane. The LamB/StxB fusion proved to be toxic for the cells and formed large intra-cytoplasmic aggregates. It is possible that either the large size of the inserted polypeptide (69 amino acids) or the strong hydrophobic domains in the C-terminal region of the B-subunit protein may affect the folding and/or assembly of the protein. Although there is a hydrophobic domain in the first 10 amino acid region of the protein it seems not to affect LamB folding and assembly since the LamB/Stx17B and LamB/Stx27B proteins were both stably expressed.

The *tac* promoter based vector for the expression of LamB hybrids, developed by Hofnung and colleagues appears to be adequate for the expression of hybrid proteins for immunization by the intra-peritoneal (i.p.) or intra-venous (i.v.) routes since smaller doses of live bacteria are required (10^5 to 10^6) as compared to oral immunization where 10^8 to 10^9 live cells are needed. Upon induction of the *tac* promoter the expression of the hybrid proteins was too high and it proved toxic to the cells making it not possible to obtain a live cell density of more than 10^7 bacteria/ml. It was therefore desireable to express the hybrid proteins using either a moderate level expression, constitutive promoter or an *in vivo* inducible promoter. This was achieved in this study by the use of a synthetic modified ß-lactamase promoter which provides moderate level, constitutive

expression and the aerobactin promoter which is induced *in vivo* under iron starvation, a condition found in the tissue of animal hosts. Indeed the *S. typhimurium aro*A- strains were stabilized with the use of these promoters and it was possible to grow the bacterial cells except for those expressing the LamB/StxB hybrid protein, to 10^9 live cells per ml of culture.

The immunization results showed that irrespective of the promoter used i.e. either the *tac* or ß-lactamase or aerobactin, significant B-subunit specific antibody responses were obtained in the intestinal fluid (secretory IgA) and in the serum (IgG and IgM). This result is expected since from western blots it was observed that all three promoters expressed significant quantities of the hybrid proteins. However, the use of ß-lactamase and aerobactin promoters significantly increased the stability of the hybrid *Salmonella* strains. A possible reason why the B-subunit specific antibody responses increased with increasing size of the B-subunit region expressed in LamB could be a reflection of epitope density which increases with size of the B-subunit. Higher antibody levels may not necessarily be as critical in protection as compared to antibodies specifically directed against the B-subunit receptor for epithelial cells or antibodies which after binding to the B-subunit would sterically hinder B-subunit interaction with the receptor.

Although ideally the expression of the candidate antigen in the *Salmonella* strain should be as high as possible to elicit maximal antibody responses, the stability of the hybrid strain is of importance. Therefore from the three systems that have been analysed in this study i.e. the utility of three different promoters, it appears that either the aerobactin or the ß-lactamase promoters could be used to express the hybrid proteins and that integration of these constructs into the chromosome of the host strain should lead to a reduction in the amount of protein expressed using multicopy vectors and thereby aleviate the residual toxicity to the host strain. This should enable the preparation of innocula of healthy live cells.

Any of the two above mentioned systems could be incorporated into candidate vaccine strains designed to elicit immune responses against *Shigella dysenteriae* 1 infections in order to also stimulate mucosal and serum antibody responses against the deleterious effects of Shiga toxin.

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Weil AJ (1943) Progress in the study of bacillary dysentery. J. Immunol. 46: 13-46.

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Figure Legends.

Table 1.

Oligonucleotides synthesised and used either for DNA sequencing, site-directed mutagenesis or PCR reaction.

Table 2.

Summary of electron microscopy analysis of whole cells of *E. coli* and *S. typhimurium* strains using either gold-labeled anti-B-subunit or anti-LamB sera. (+ and -) indicates that the respective region (either B-subunit or LamB) was detected or not on the bacterial cell surface.

Fig. 1

Plasmid construction for high level expression of B-subunit and generation of LamB/B subunit fusions. For several plasmids only the insert region with relevant vector details are shown.

Fig. 2.1

Commassie blue stained SDS-PAGE analyses of high level expression and purification of B-subunit. Lane A: molecular weight markers; lane B: heat denatured bacterial extract from strain DH5/pJLA503; lanes C and D: heat-denatured bacterial extracts from strain DH5/pSU108 before and after induction with IPTG respectively; lanes E and F: After osmotic shock treatment of strain DH5/pSU108, the pellet and supernatant fractions respectively; lane G: FPLC purified B-subunit from the supernatant fraction shown in lane F. The premature and mature forms of the B- subunit are shown by arrows.

Fig. 2.2

Autoradiograph of western blot showing reactivity of B-subunit specific polyclonal antiserum with B subunit. IPTG induced, heat-denatured bacterial extracts were electrophoresed on SDS-PAGE. Lane A: strain DH5/pSU108; lane B: strain DH5/pJLA503.

Fig. 2.3

Autoradiograph of western blot showing reactivity of StxBMb1 monoclonal antibody with B-subunit. IPTG induced, heat-denatured bacterial extracts were electrophoresed on SDS-PAGE. Lane A: strain DH5/pJLA503; lane B: strain DH5/pSU108.

Fig. 3

Autoradiograph of western blot showing expression of LamB/StxB, LamB/Stx27B and LamB/Stx17B in *E. coli* pop6510 (Fig. 3A) and *S. typhimurium* SL3235 (Fig. 3B). Heat denatured bacterial extracts were electrophoresed on SDS-PAGE. The lanes are in pairs showing expression before and after induction with IPTG. The proteins were revealed using the preabsorbed B-subunit polyclonal antiserum. Lanes 1 and 2: host strain/pAJC264; lanes 3 and 4: host strain/pSU112 (LamB/StxB); lanes 5 and 6: host strain/pSU113 (LamB/Stx27B); lanes 7 and 8: host strain /pSU114.

The Bio-Rad molecular weight markers are shown (a to f; see materials and methods for values).

Fig. 4

Electron microscopy analysis of whole cells (panel A) or thin bacterial sections (panel B) of *E. coli* K-12 strain pop6510 (Fig. 4.1), *S. typhimurium* strain SL3235 (Fig. 4.2), *S. typhimurium* strain SL5283 (with no defects in *aro* genes) (Fig. 4.3) and *E. coli aro*A- strain AB2826 (Fig. 4.4). The primary antibody used in the labeling was gold-labeled anti-B subunit antibodies. (a and e) host strain/pAJC264, (b and f) host strain/pSU112 (LamB/StxB), (c and g) host strain/pSU113 (LamB/Stx27B), (d and h) host strain/pSU114 (LamB/Stx17B). Fig. 4.5 shows indirect immunofluorescence analysis of *E. coli* K-12 (pop6510) cells expressing LamB/B-subunit hybrid proteins. The same field is shown under (a) under phase contrast and (b) immunofluorescence.

Fig. 5

Nucleotide sequences of the native ß-lactamase promoter (A) and the modified synthetic version (B). The -35 and -10 regions, the +1 transcriptional start site, the ribosome binding site (RBS) and the ATG translation start codon are shown. The newly introduced *Eco*RI, *Xho*I, *Nde*I, and *Bam*HI restriction enzyme sites are also indicated.

Fig. 6

Construction of;

(i) Function tester plasmid for ß-lactamase promoter (pSU208).

(ii) An intermediate plasmid (pSU207) for replacement of the *tac* promoter in LamB/B-subunit constructs with the aerobactin promoter. (iii) Plasmid for the replacement of the *tac* promoter in LamB/B-subunit plasmids with a ß-lactamase and aerobactin promoters.

Fig. 7

Autoradiograph of western blot showing expression of B-subunit/ß-galactosidase fusion protein under the control of aerobactin promoter in plasmid pSU207 in *Salmonella typhimurium aro*A- strains SL3235 and SL3261. Heat-denatured bacterial extracts are electrophoresed and the fusion proteins are revealed by B-subunit specific polyclonal antiserum. Lanes shown are in pairs before and after induction with dipyridyl. Lanes 1 and 2: strain pcon1/SL3261 as a negative control; lanes 3 and 4: strain pSU207/SL3261; lanes 5 and 6: strain pSU207/SL3235. The first four bands of the Bio-Rad molecular weight markers are indicated.

Fig 8

Autoradiograph of western blot showing expression of LamB/B-subunit fusions under the expression of modified, synthetic ß-lactamase promoter.

(A) All plasmids are harboured in *E. coli* strain pop6510 and the bands are revealed with polyclonal anti-LamB antiserum. The Bio-Rad molecular weight marker positions are indicated on the left. Lane 1: pop6510; lane 2: pSU116 (ß-*lacP*/*lam*B); lane 3: pSU119 (ß-*lacP*/*lam*B/*stx*17B); lane 4: pSU118 (ß-*lacP*/*lam*B/*stx*27B); lane 5: pSU117 (ß-*lacP*/*lam*B/*stx*B).

(B) All plasmids are harboured in *S. typhimurium aro*A- strain SL3261 and the bands are revealed with polyclonal B-subunit antiserum. The Sigma molecular weight marker positions are indicated on the left. Lane 1: pSU116; lane 2: pSU119; lane 3: pSU118; lane 4: pSU117.

Fig. 9

Autoradiograph of western blot showing expression of LamB/B-subunit fusions under the expression of the aerobactin promoter. All plasmids are harboured in *E. coli* strain pop6510. Gels (A) and (B) have been developed using polyclonal LamB antiserum and polyclonal B-subunit antiserum respectively. The lanes are shown in pairs, uninduced culture and induced with dipyridyl respectively. Lanes 1 and 2: pSU121 (*aroP*/*lamB*); lanes 3 and 4: pSU124 (*aroP*/*lamB*/*stx*17B); lanes 5 and 6: pSU123 (*aroP*/*lamB*/*stx*27B); lanes 7 and 8: pSU122 (*aroP*/*lamB*/*stx*B).

Fig. 10

Serum IgG+IgM and mucosal IgA B-subunit specific responses after oral and i.p. immunizations of BALB/c mice with various *Salmonella typhimurium aro*A- hybrid strains expressing LamB/B-subunit proteins. Each bar represents the mean of ELISA units determined from four mice. Standard deviation bars are shown. Serum and mucosal B-subunit responses were zero for control animals.

Fig. 10.1.

B-subunit responses using *S. typhimurium aro*A- strain (SL3261) carrying plasmids pSU112 (*tacP*/*lamB*/*stx*B), pSU113 (*tacP*/*lamB*/*stx*27B), pSU114 (*tacP*/*lamB*/*stx*17B) and pAJC264 (*tacP*/*lamB*; negative control).

Fig. 10.2.

B-subunit responses using *S. typhimurium aro*A- (SL3261) carrying plasmids pSU117 (ß-*lacP*/*lamB*/*stx*B), pSU118 (ß-*lacP*/*lamB*/*stx*27B), pSU119 (ß-*lacP*/*lamB*/*stx*17B) and pSU116 (ß-*lacP*/*lamB*; negative control).

Fig. 10.3.

B-subunit responses using *S. typhimurium aro*A- (SL3261) carrying plasmids pSU122 (*aroP*/*lamB*/*stx*B), pSU123 (*aroP*/*lamB*/*stx*27B), pSU124 (*aroP*/*lamB*/*stx*17B) and pSU121 (*aroP*/*lamB*; negative control).

Table 1

Oligonucleotide	DNA sequence
StxB-N	Pro Thr +2 +1 BamHI 5′ AATCAGGCGTCGGATCCGCACTTGCTGA 3′
StxB-C	Ser Phe BglII +69 +68 5′ CTGAGCTATTCTGAGATCTCGAAAAATAAC 3′
StxB-3	lys Asp BglII +27 +26 5′ TGTTGGTAAATAGATCTTTATCACCC 3′
LamB-8	5′ CTCAGGAGATCTAATGATGATTACTCTGCTC 3′
LamB-9	5′ GCCAACATCGATGTTTTCCAG 3′

	Antisera		
Strain	LamB/B subunit fusion	anti-B subunit polyclonal	anti-LamB
E. coli K-12 pop6510	LamB	–	+
	LamB/Stx17B	+	+
	LamB/Stx27B	+	+
	LamB/StxB	–	+
E. coli aro A-AB2826	LamB	–	+
	LamB/Stx17B	+	+
	LamB/Stx27B	+	+
	LamB/StxB	–	+
S. typhimurium aro A-SL3235	LamB	–	–
	LamB/Stx17B	–	–
	LamB/Stx27B	–	–
	LamB/StxB	–	–
S. typhimurium aro A-SL3261	LamB	–	–
	LamB/Stx17B	–	–
	LamB/Stx27B	–	–
	LamB/StxB	–	–
S. typhimurium SL5283	LamB	–	+
	LamB/Stx17B	+	+
	LamB/Stx27B	+	+
	LamB/StxB	–	+

Table 2

Claims

1. Dysentery vaccine consisting of or comprising a component which stimulates an immune response against Shiga toxin, **characterized** in that the said component is selected from microorganisms expressing the Shiga toxin B subunit or a fragment or a derivative of the said B subunit.

2. Dysentery vaccine according to claim 1, **characterized** in that the microorganisms express the Shiga toxin B subunit or a fragment or a derivative of the said B subunit in the form of a hybrid protein.

3. Dysentery vaccine according to claim 2, **characterized** in that the hybrid protein contains a protein selected from the Lam B protein and its fragments comprising at least the cell surface exposed loop of Lam B.

4. Dysentery vaccine according to claim 2 or 3, **characterized** in that the expression of the hybrid protein is effected by a promoter system.

5. Dysentery vaccine according to claim 4, **characterized** in that the promoter system is selected from the group comprising the tac promoter, the aerobactin promoter and a modified synthetic β-lactamase promoter.

6. Dysentery vaccine according to any of the preceding claims, **characterized** in that the microorganisms are selected from the group comprising E. coli and Salmonella typhimurium bacteria.

7. Dysentery vaccine according to claim 6, **characterized** in that the microorganisms are selected from E. coli pop 6510, S. typhimurium SL 3235, S. typhimurium SL 5283, E. coli AB 2829 and S. typhimurium SL 3261.

8. Dysentery vaccine according to any of the preceding claims, **characterized** in that the said fragments of the Shiga toxin B subunit comprise the N-terminal amino acids x to 27, x being an integer from 1 to 11, and especially the N-terminal 27 amino acids or the N-terminal amino acids 11-27.

9. Dysentery vaccine according to any of the preceding claims, **characterized** in that the microorganisms contain as their expressing system a plasmid selected from pSU 112, 113, 114, 117, 118, 119, 122, 123, and 124.

10. Dysentery vaccine according to any of the preceding claims, **characterized** in that the microorganisms are selected from E. coli pop 6510/pSU112, E. coli pop 6510/pSU 113, E. coli pop 6510/pSU 114, S. typhimurium SL 3235/pSU 112, S. typhimurium SL 3235/pSU 113, S. typhimurium SL 3235/pSU 114, S. typhimurium SL 3261/pSU 112, S. typhimurium SL 3261/pSU 113, S. typhimurium SL3261/pSU 114, S. typhimurium SL 3261/pSU 117, S. typhimurium SL 3261/pSU 118, S. typhimurium SL 3261/pSU 119, S. typhimurium SL 3261/pSU 122, S. typhimurium SL 3261/pSU 123, and S. typhimurium SL 3261/pSU 124.

11. Method for preparing a dysentery vaccine according to any of claims 1 to 10, **characterized** in that the microorganism is prepared using the following steps:

- a plasmid carrying a gene encoding the Shiga toxin B subunit or a fragment or a derivative of the said B subunit which optionally carries one or more restriction sites on the said gene is digested and religated into the BamHI site of pAJC264,

- optionally, the resulting plasmid is further cleaved at a restriction site on the said gene and religated to eliminate parts of the said gene,

- the resulting plasmid is transformed into a host which serves as the said microorganism.

12. Method according to claim 11, **characterized** in that the plasmid carrying a gene encoding the Shiga toxin B subunit is pSU 110 or pSU 111.

13. Method according to claim 11 or 12, **characterized** in that the resulting plasmid is pSU 112, pSU 113, or pSU 114.

14. Method according to any of claims 11 to 13, further **characterized** in that prior to its transformation the resulting plasmid is cleaved and religated into a plasmid carrying a β-lactamase promoter or an aerobactin promoter.

15. Method according to claim 14, **characterized** in that the plasmid carrying the β-lactamase or aerobactin promoter further contains a PCR-fragment encoding part of the signal sequence of Lam B, synthesized as a BglII/ClaI PCR-fragment.

16. Method according to claim 15, **characterized** in that the PCR-fragment comprises the structure

5' CTCAGGAGATCTAATGATGATTACTCTGCTC 3'

or the structure

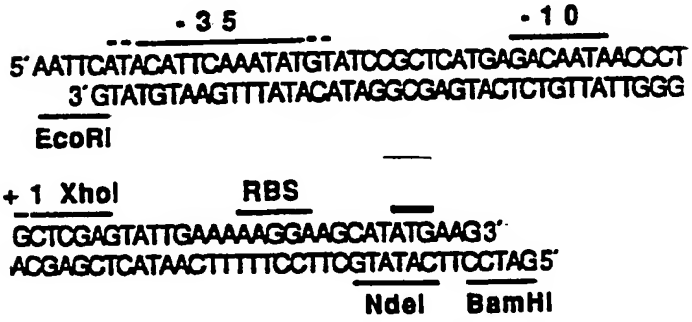
5' GCCAACATCGATGTTTTCCAG 3'

or a DNA sequence that can be hybridized with one of the foregoing two structures at a temperature of at least 25 °C and at a 1 M concentration of NaCl and that enables the plasmid

according to claim 15 comprising said DNA sequence as PCR fragment to express the Shiga toxin B subunit or a fragment or a derivative of the said B subunit.

17. Method according to any of claims 14 to 16, **characterized** in that the ß-lactamase promoter is a non-wild type ß-lactamase promoter.

18. Method according to claim 17, **characterized** in that the ß-lactamase promoter comprises the following sequence:



or a DNA sequence that hybridizes with the foregoing sequence at a temperature of at least 25 °C and at a 1 M concentration of NaCl and that enables the plasmid according to claim 15 comprising said DNA sequence as PCR fragment to express the Shiga toxin B subunit or a fragment or a derivative of the said B subunit.

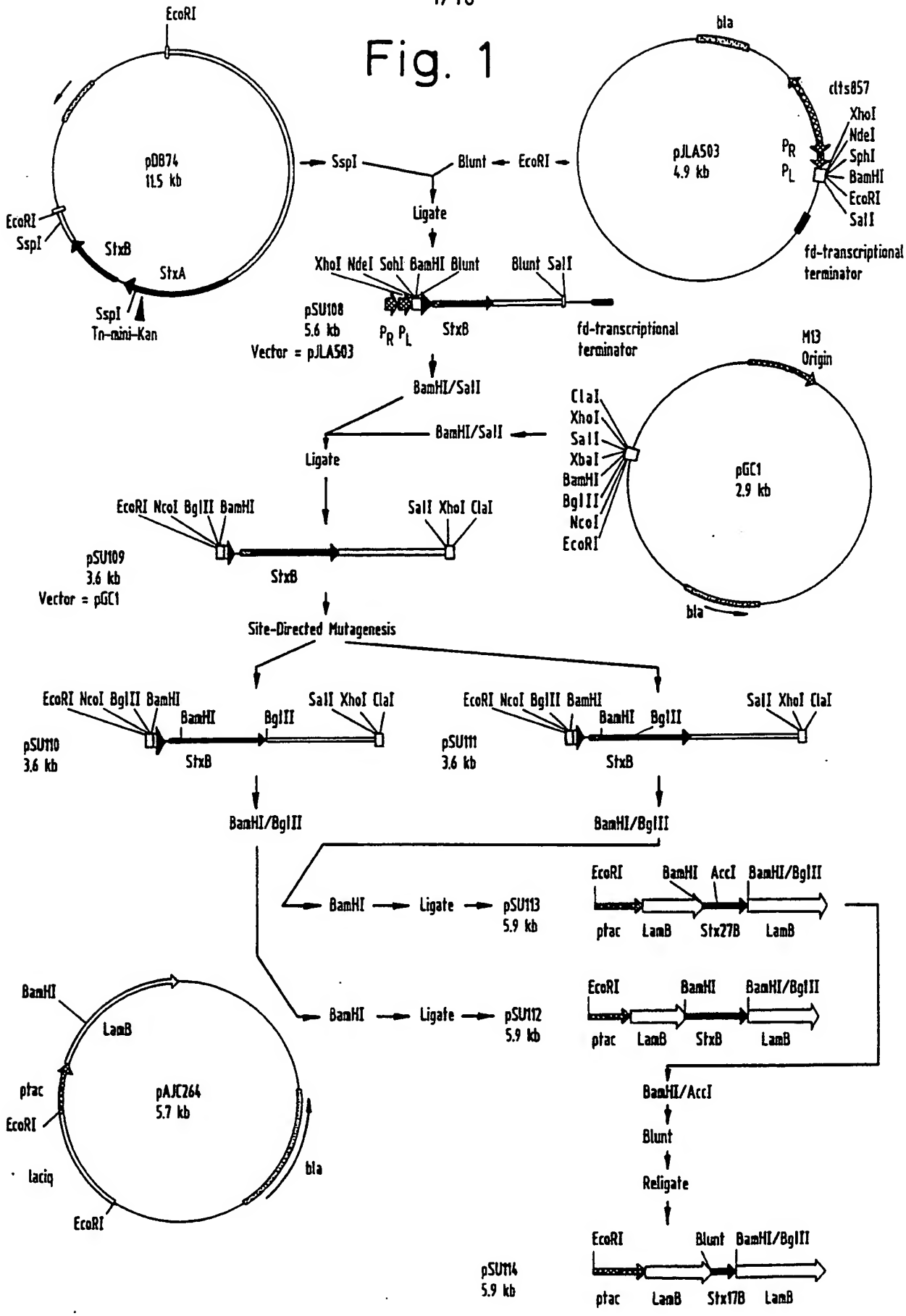
19. Method according to any of claims 14 to 18, **characterized** in that the plasmid carrying the ß-lactamase promoter is pSU 115 or the plasmid carrying the aerobactin promoter is pSU 120.

20. Method according to any of claims 11 to 19, **characterized** in that the resulting plasmid is transformed into a host, selected from the group comprising E. coli K-12 pop 6510, E. coli AB 2826, S. typhimurium SL 3235, S. typhimurium SL 3261, and S. typhimurium SL 5283.

21. pSU 112, pSU 113, pSU 114, pSU 115, pSU 117, pSU 118, pSU 119, pSU 120, pSU 122, pSU 123 and pSU 124, E. coli pop 6510/pSU 112, E. coli pop 6510/pSU 113, E. coli pop 6510/pSU 114, S. typhimurium SL 3235/pSU 112, S. typhimurium SL 3235/pSU 113, S. typhimurium SL 3235/pSU 114, S. typhimurium SL 3261/pSU 112, S. typhimurium SL 3261/pSU 113, S. typhimurium SL 3261/pSU 114, S. typhimurium SL 3261/pSU 117, S. typhimurium SL 3261/pSU 118, S. typhimurium SL 3261/pSU 119, S. typhimurium SL 3261/pSU 122, SL typhimurium SL 3261/pSU 123, and S. typhimurium SL 3261/pSU 124.

22. The gene products expressed by hosts, bacteria, plasmids or systems according to any of claims 11 to 21.

Fig. 1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NdeI
SphI
BamHI
EcoRI
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P_L
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Ligate
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Blunt SalI
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StxB
fd-transcriptional terminator
BamHI/SalI
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Ligate
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XhoI
SalI
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Fig. 2

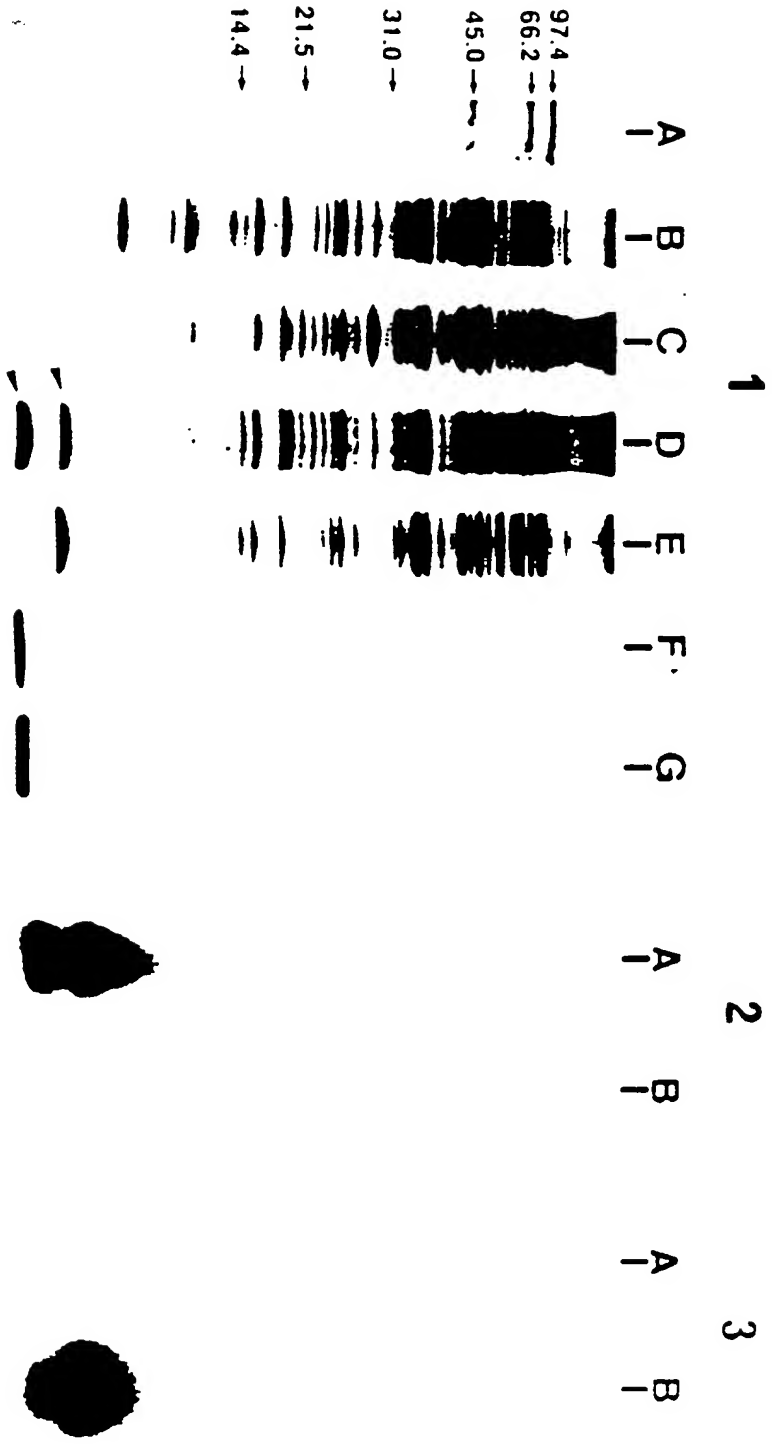

Fig. 3

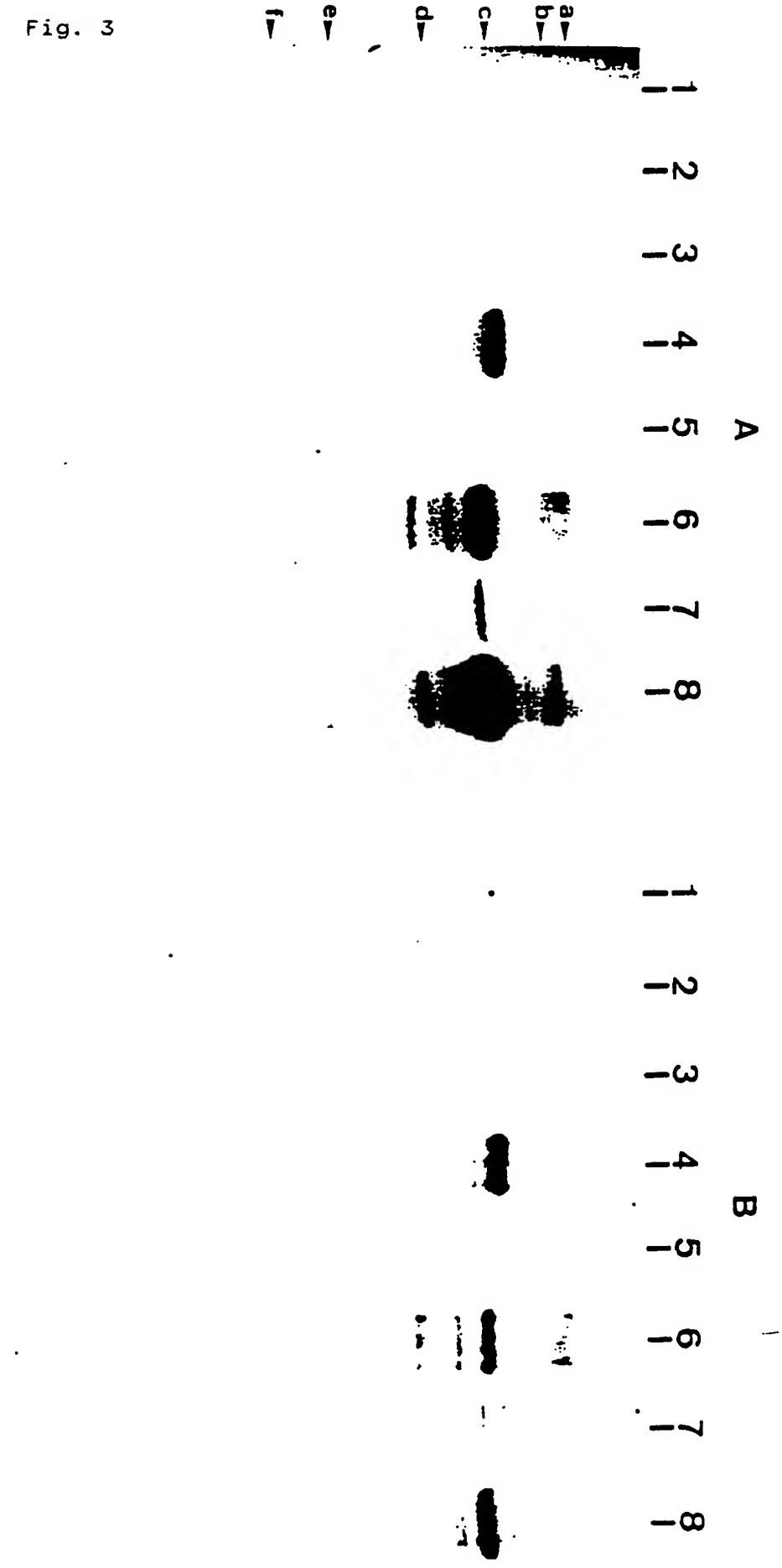

Fig. 4.1

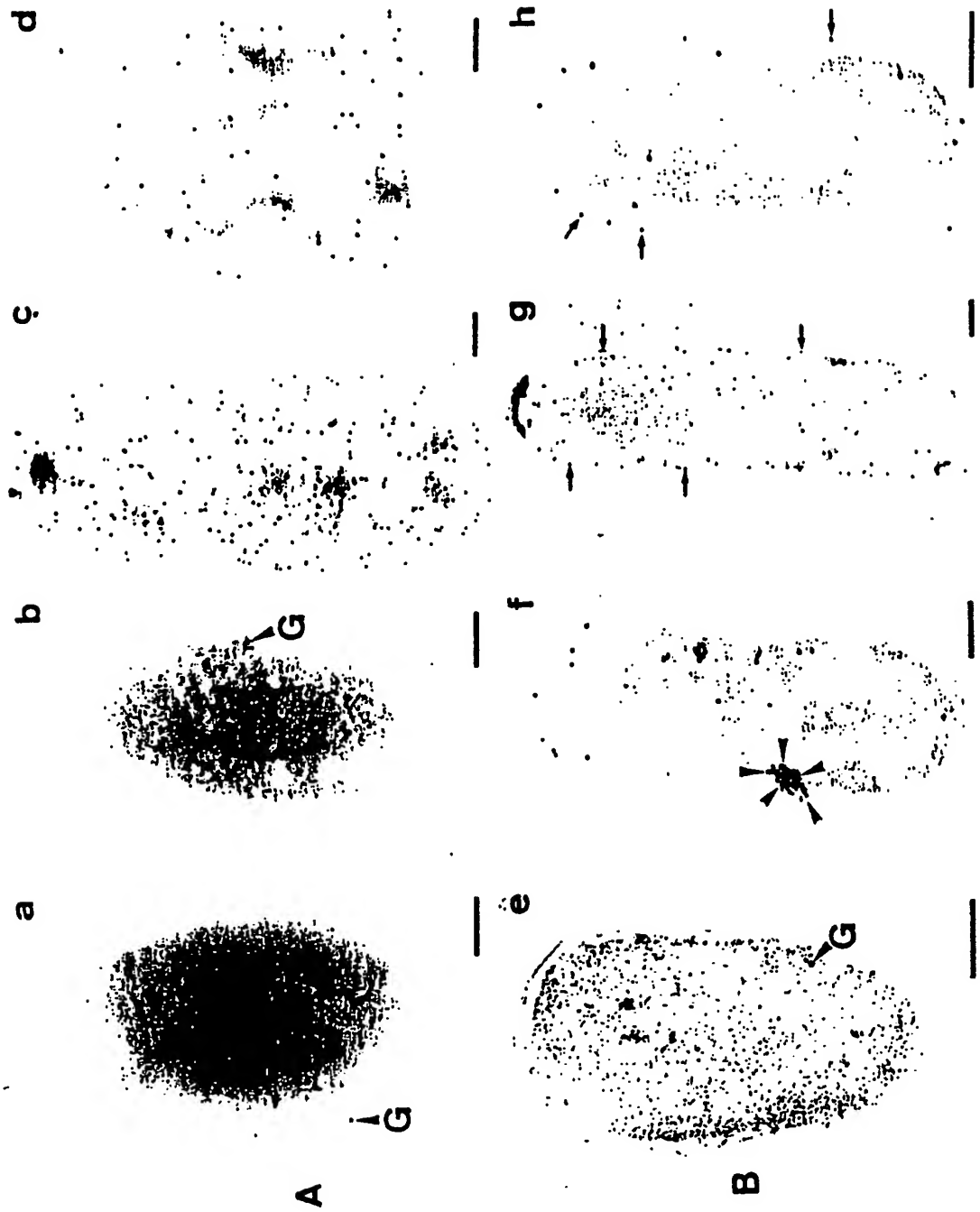


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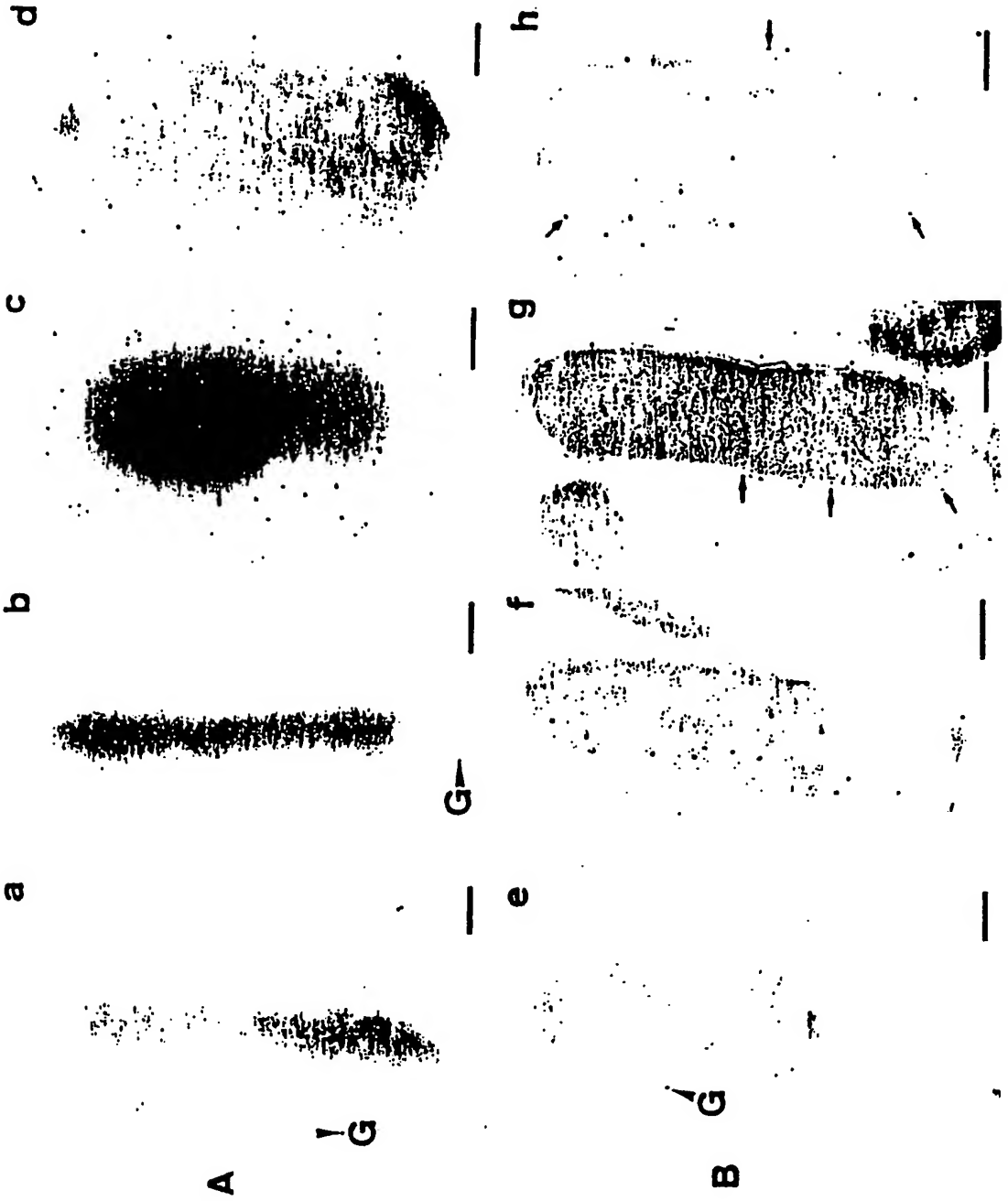
Fig. 4.3 6/16



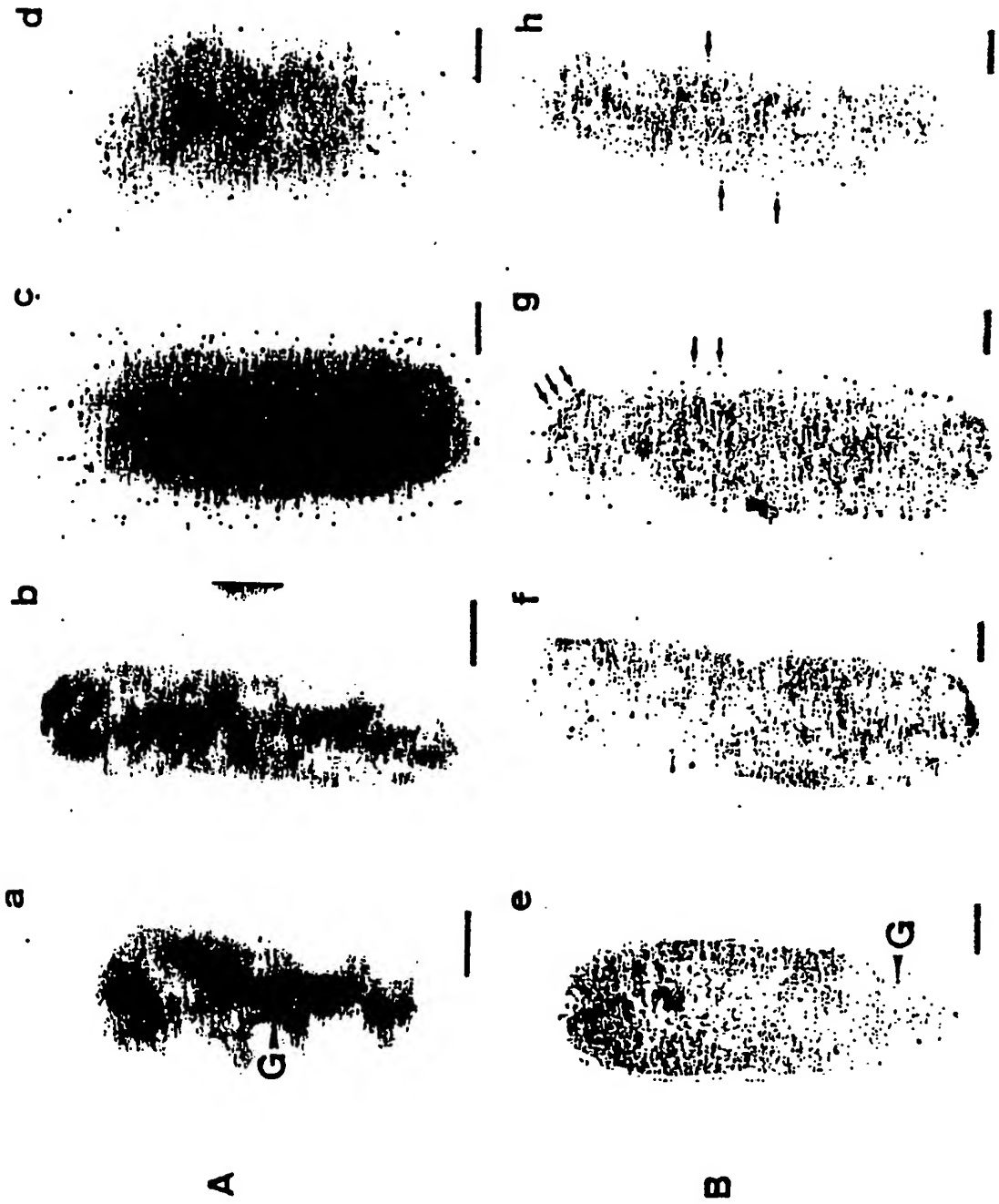

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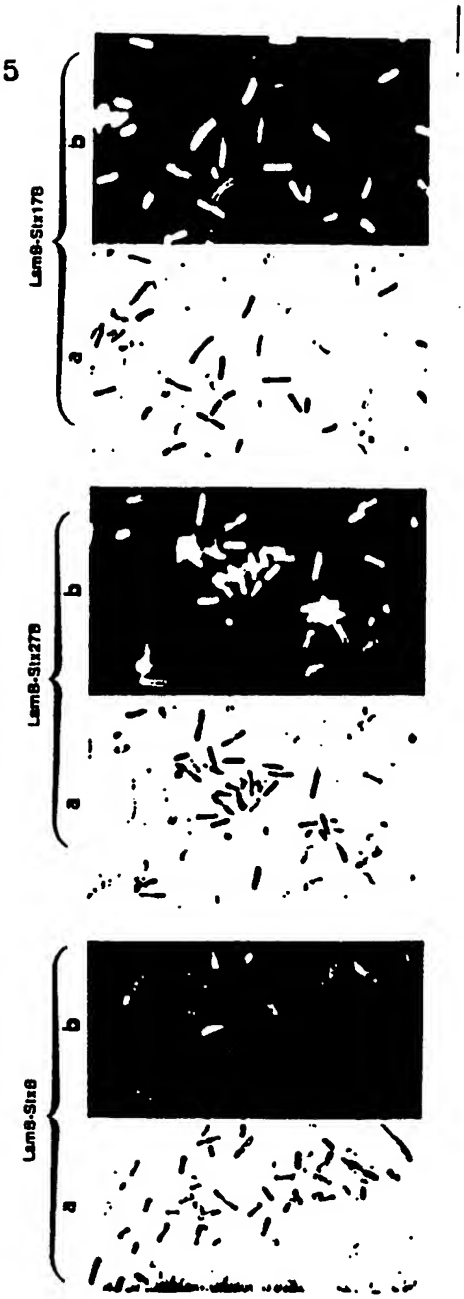
Fig. 4.5



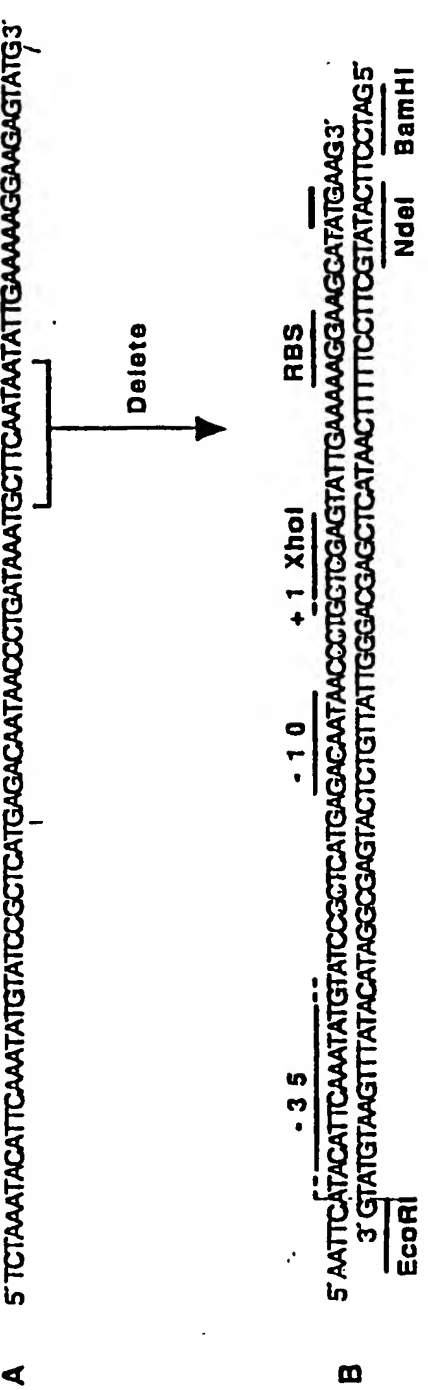

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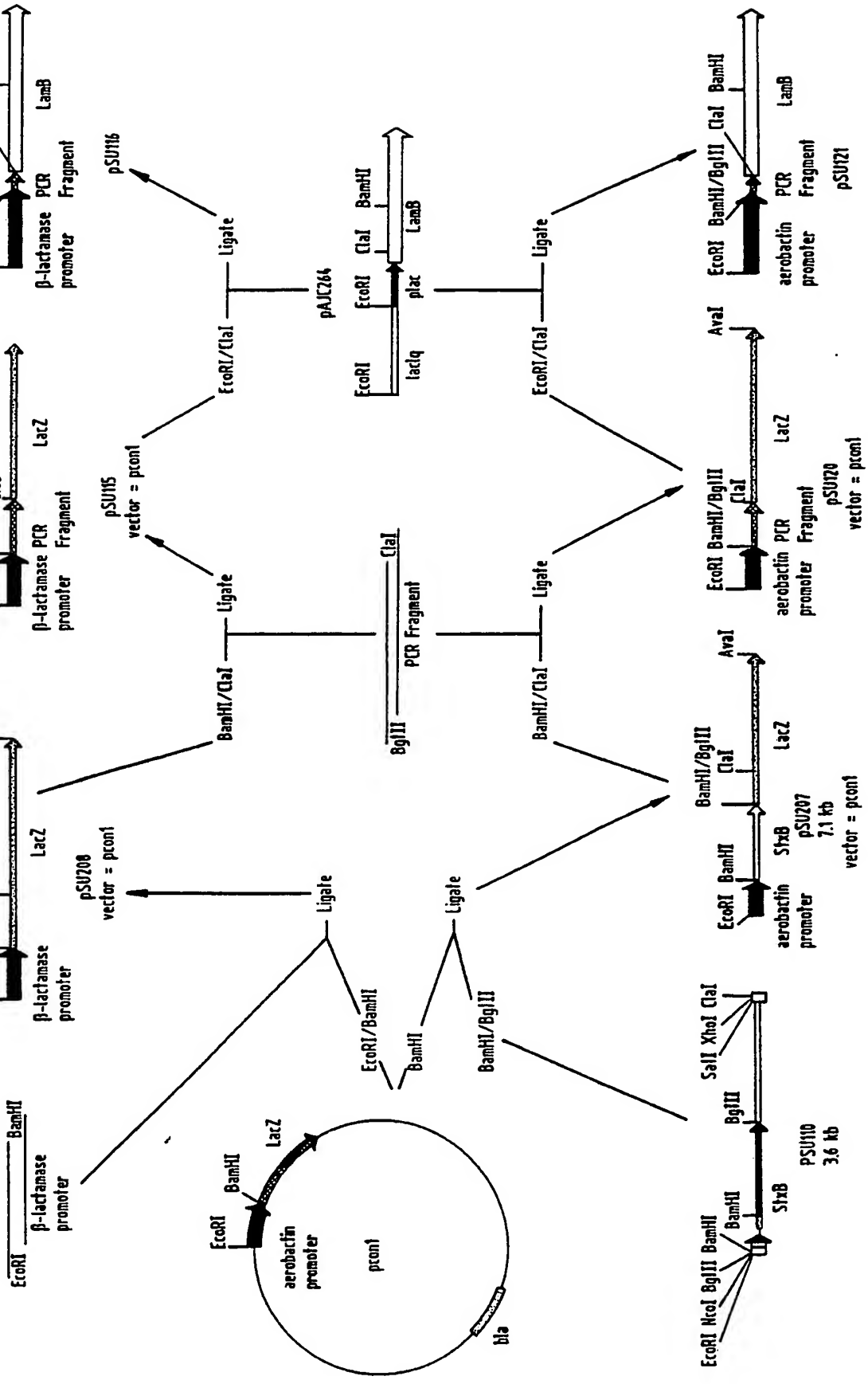
Fig. 5

Fig. 6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β-lactamase promoter
LacZ
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lacIq
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ClaI
BamHI
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aerobactin promoter
PCR Fragment
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Fig. 7

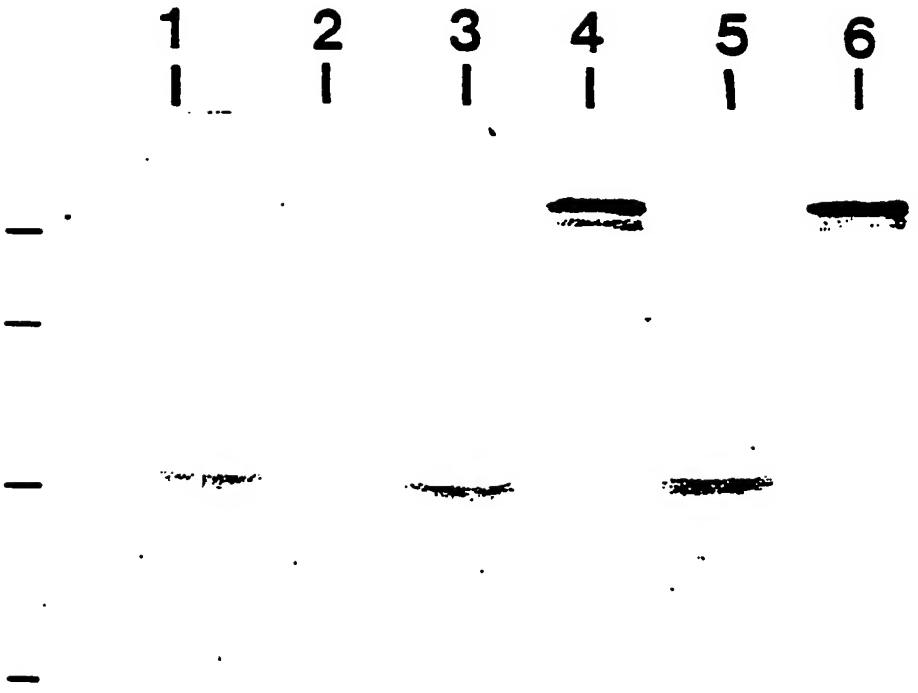


Fig. 8

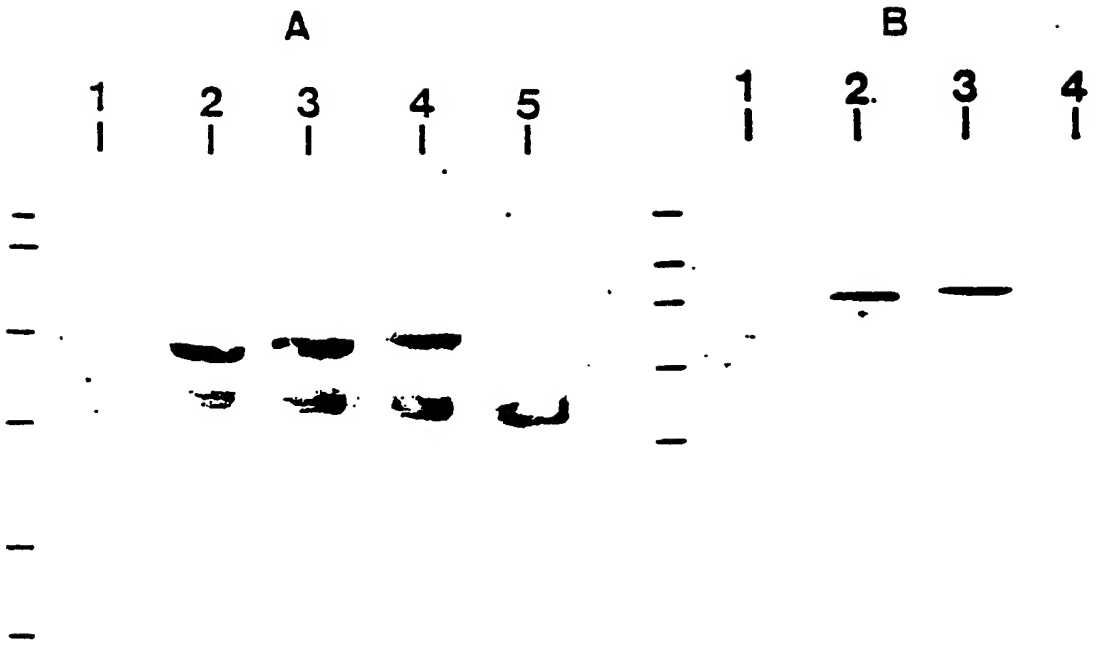


Fig. 9

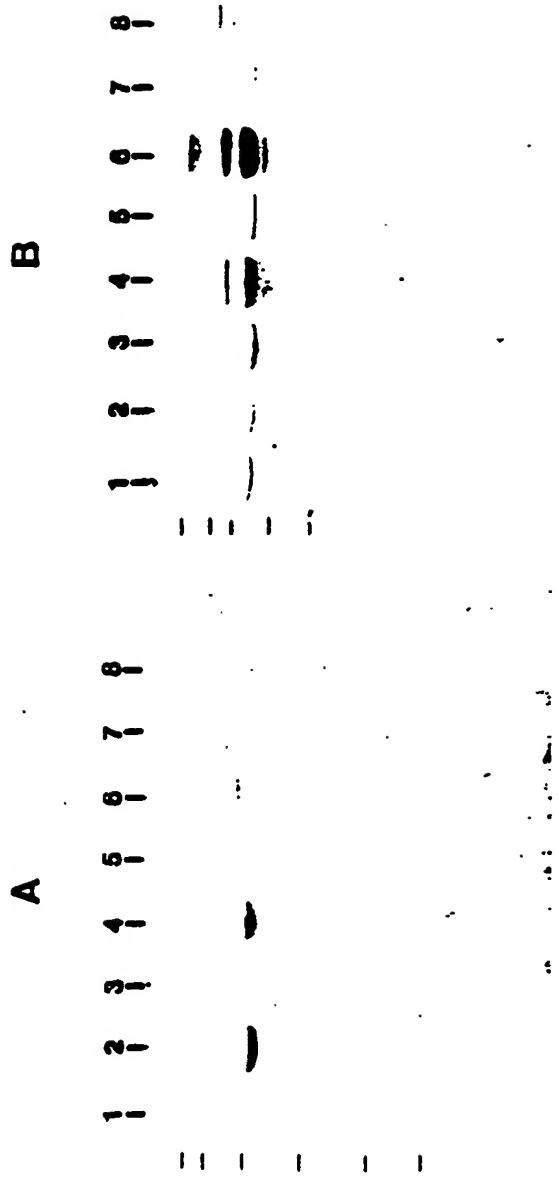
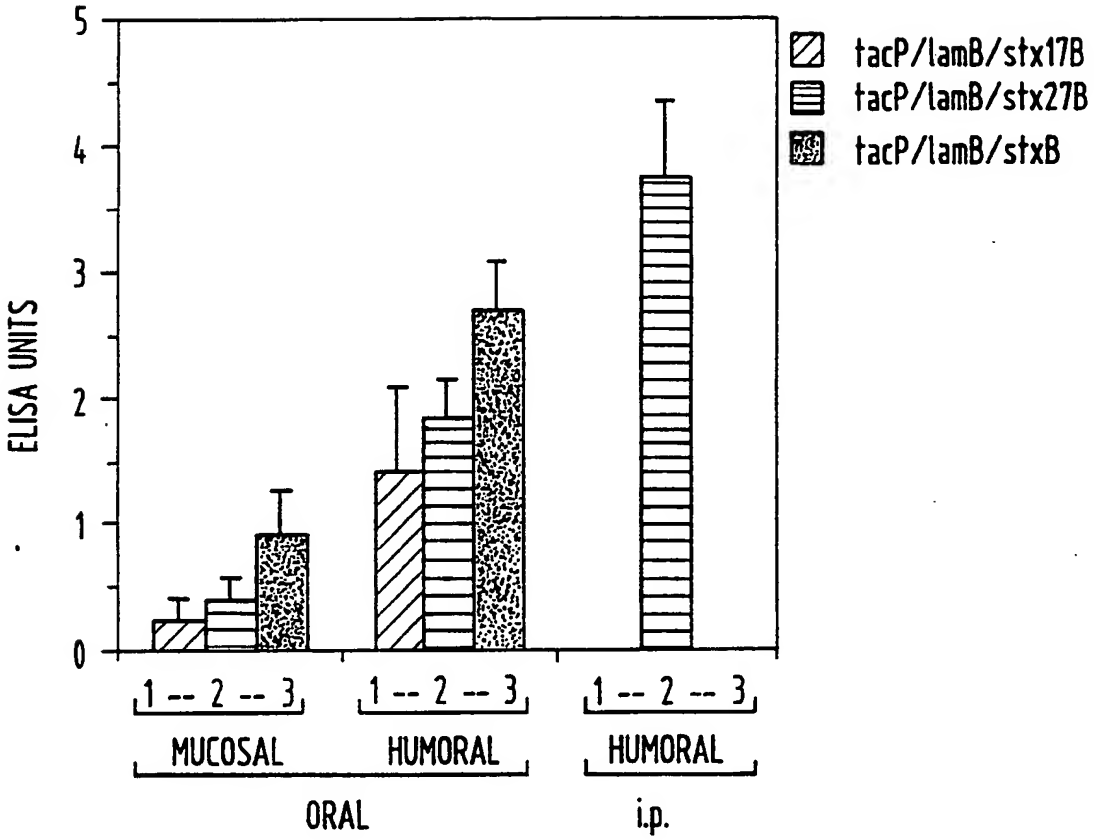


Fig. 10.1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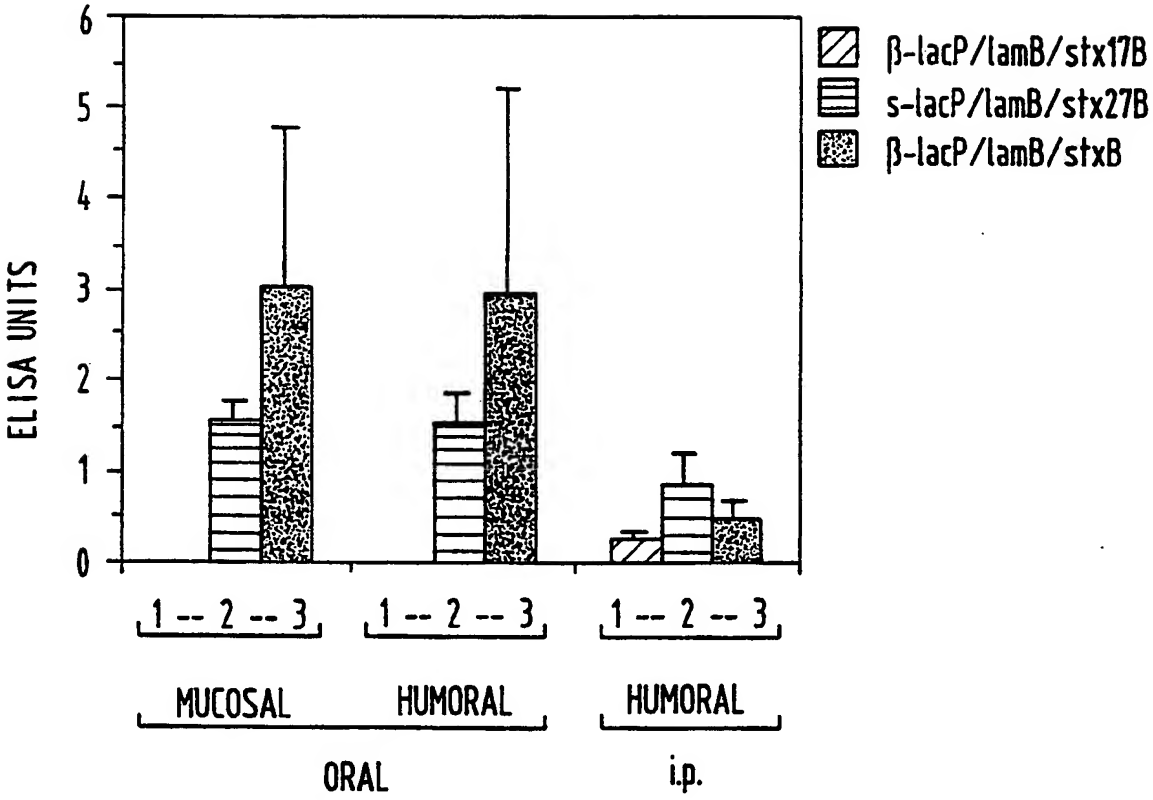
HUMORAL
ORAL
i.p.

Fig. 10.2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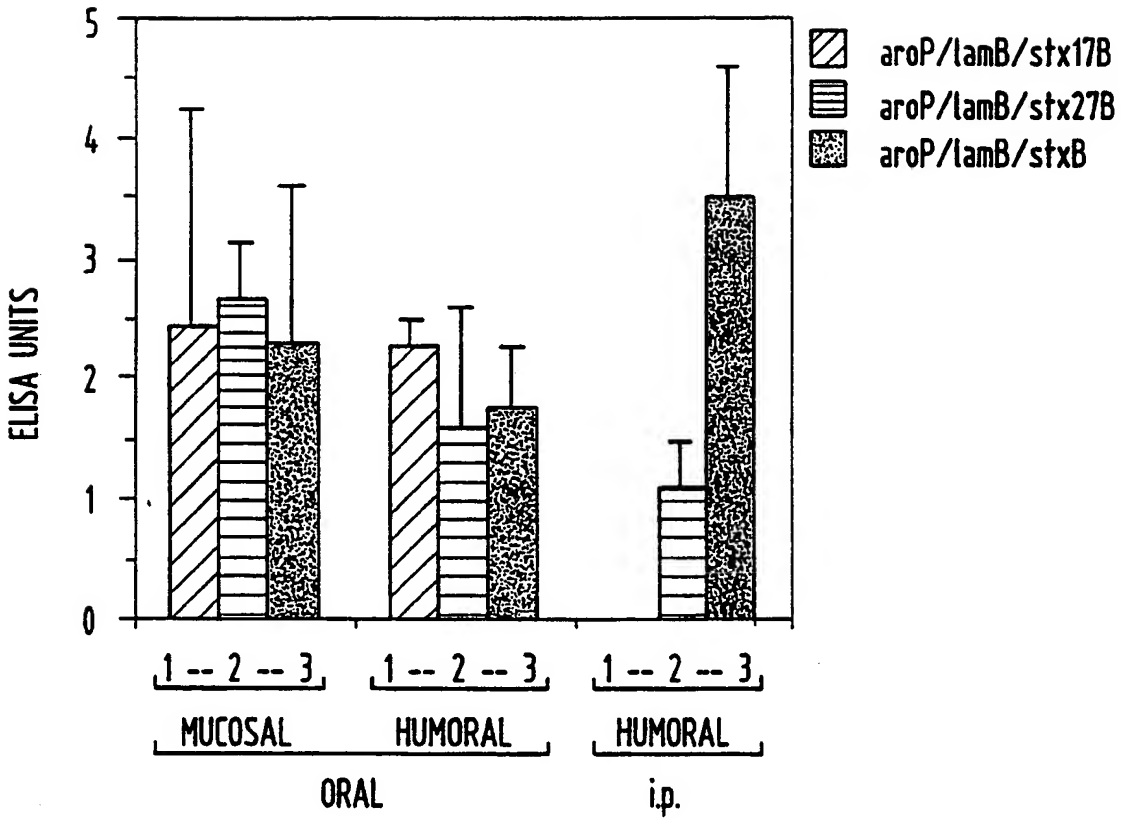
ORAL
i.p.

Fig. 10.3


ELISA UNITS
0
1
2
3
4
5
1 -- 2 -- 3
1 -- 2 -- 3
1 -- 2 -- 3
MUCOSAL
HUMORAL
HUMORAL
ORAL
i.p.
aroP/lamB/stx17B
aroP/lamB/stx27B
aroP/lamB/stxB